Photoperiodic and diurnal regulation of WNT signalling in the arcuate nucleus of the female Djungarian hamster, *Phodopus sungorus*

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Abstract

The WNT pathway was shown to play an important role in the adult central nervous system. We previously identified the WNT pathway as a novel integration site of the adipokine leptin in mediating its neuroendocrine control of metabolism in obese mice. Here, we investigated the implication of WNT signalling in seasonal body weight regulation exhibited by the Djungarian hamster (*Phodopus sungorus*), a seasonal mammal that exhibits profound annual changes in leptin sensitivity. We furthermore investigated whether crucial components of the WNT pathway are regulated in a diurnal manner. Gene expression of key components of the WNT pathway in the hypothalamus of hamsters acclimated to either long day (LD) or short day (SD) photoperiod was analysed by *in-situ*-hybridization. We detected elevated expression of the genes WNT-4, Axin-2, Cyclin-D1 and SFRP-2, in the hypothalamic arcuate nucleus, a key energy balance integration site, during LD compared with SD, as well as a diurnal regulation of Axin-2, Cyclin-D1 and DKK-3. Investigating the effect of photoperiod as well as leptin on the activation (phosphorylation) of the WNT coreceptor LRP-6-(Ser1490) by immunohistochemistry, we found elevated activity in the arcuate nucleus during LD relative to SD, as well as after leptin treatment (2 mg/kg body weight). These findings indicate that differential WNT signalling may be associated with seasonal body weight regulation and is partially regulated in a diurnal manner in the adult brain. Furthermore, they suggest that this pathway plays a key role in the neuroendocrine regulation of body weight and integration of the leptin signal.

Introduction

Survival in a seasonally changing environment requires physiological adaptations for most animals. In seasonal mammals, these adaptations are driven by photoperiod and exhibit remarkable changes in growth, energy balance and reproduction, resulting from changes in the neuroendocrine axis.

During the last decade, significant progress has been achieved in unravelling the molecular mechanisms underlying these physiological changes. One of the best characterized seasonal rodent models is the Djungarian hamster (*Phodopus sungorus*), also known as the Siberian hamster, as these mammals show an extensive body weight reduction of about 40 % in winter-like conditions (short days; SD) compared with summer-like conditions (long days; LD). More than half of this weight loss derives from a reduction of adipose tissue mass (1). This annual body weight cycle is characterized by seasonally regulated leptin sensitivity (1).

Accumulating evidence suggests that the WNT signalling pathway, which has been well characterized in embryogenesis and tumorigenesis (2,3) can be altered in diverse tissues by the body weight regulating hormone leptin (4,5). Additionally, hypothalamic WNT signalling was shown to be implicated in the control of adipogenesis, adult neurogenesis as well as the cellular and structural remodelling of the adult hypothalamus (6-8). Recent findings from our laboratory suggested that the central WNT pathway is crucial for the neuroendocrine control of metabolism in mice (5,9). Furthermore, several genes involved in WNT signal transduction were shown to be regulated by photoperiod in photoperiod-responsive F344 rats (8,10,11).

The canonical, β-catenin-dependent WNT pathway is activated when extracellular WNT ligands bind to Frizzled (Fz) receptors and associated coreceptors, the low-density lipoprotein receptor-related proteins (LRPs). The coreceptor LRP-6 is activated via phosphorylation at Ser1490 (12). Subsequently, the formation of a protein complex including Axin-1, Dishevelled and adenomatous polyposis coli is prevented, resulting in an inhibition of the key enzyme glycogen synthase kinase-3β (GSK-3β). This leads to the stabilization of the co-transcription factor β-catenin in the cytoplasm and its translocation to the nucleus, where it promotes binding of the transcription factor T cell-specific transcription factor 7 (TCF-7), which then induces the expression of WNT target genes (13). In the

absence of WNT ligands (WNTs) or in the presence of WNT antagonists such as Dickkopf (DKK) proteins or secreted Fz-related proteins (SFRPs), GSK-3β lingers in the complex and phosphorylates β-catenin, inducing its proteasomal degradation (13).

Circadian clocks adjust behavioural and physiological processes to the most beneficial time of day in a broad range of species. In mammals, the primary entrainment signal ("Zeitgeber") is light, which synchronizes the circadian clock, whose pacemaker resides in the hypothalamic suprachiasmatic nucleus (SCN) with environmental cues (14). The circadian clock is tightly coupled to metabolism and feeding rhythms (15,16) and GSK-3β has been described as being a crucial part of the transcriptional-translational feedback loop that comprises the clock, since this kinase is responsible for phosphorylation of key clock components and might thereby directly affect circadian rhythms (17-21). Furthermore, the prominent WNT target gene Cyclin-D1 was shown to be under control of the circadian clock in the periphery (22). Most of our knowledge about the circadian regulation of WNT signalling derives from stem cell proliferation and cancer studies. However, whether WNT signalling molecules are regulated in a diurnal manner in the hypothalamus is unknown.

In the current study we analysed whether WNT signalling is active in the hypothalamus of adult Djungarian hamsters and is furthermore regulated by photoperiod or in a diurnal manner, potentially implicating a novel regulatory role in the hamsters' profound seasonal alterations in physiology. We therefore examined seasonal as well as diurnal regulation of the WNT pathway by analysing whether key molecules of the WNT pathway are differentially regulated, on a transcriptional and post-translational level, between hamsters held in LD and SD at different times throughout the day. Furthermore, we challenged hamsters with leptin administration to corroborate our recent finding that leptin activates the WNT pathway in a different rodent species (5).

Materials and methods

Animals

All procedures involving animals were performed in accordance with German animal ethics legislation and received approval by the respective authorities for animal ethics. Female Djungarian hamsters (*Phodopus sungorus*) were bred under LD conditions (light/dark cycle: 16 h light/8 h dark) at the Biology Department of the University of Marburg (Marburg, Germany). At the age of 3 weeks, hamsters were weaned and housed individually at an ambient temperature of 21 °C with *ad libitum* access to standard chow diet and water. Hamsters were maintained in LD or, where specified, transferred to SD conditions (light/dark cycle: 8 h light/16 h dark) for further 8 weeks until they were fully adapted to SD. Body weight was recorded weekly during this time. Before animals were killed, they were food deprived for 16 hours. For *in situ* hybridization, animals were euthanized by cervical dislocation and brains were rapidly frozen on dry ice. For immunohistochemistry, transcardial perfusion was performed and brains were treated as described elsewhere (23). To characterise diurnal expression profiles of key WNT genes adult Djungarian hamsters were used. After weaning, all hamsters were maintained in LD until adulthood (3–5 months of age), whereupon they either remained in LD or were transferred to SD for a further 8 weeks. During LD, the light period was from Zeitgeber time (ZT) 0 to ZT16, during SD from ZT0 to ZT8. Hamsters were killed at ZT0, ZT3, ZT6, ZT9, ZT12, ZT15, ZT18, and ZT21 (n = 4–5 animals per photoperiod and time point). Hamsters killed during the dark phase were euthanized under dim red light.

Detection of hypothalamic gene expression by *in situ* hybridization

In situ hybridization was performed on brains of Djungarian hamsters aged 5–7 months. Coronal brain sections (16 μm) were collected throughout the extent of the arcuate nucleus (ARC) onto a set of twelve slides with ten sections mounted on each slide, as described previously (24). The slides spanned the hypothalamic region approximately from −2.7 to −0.8 mm relative to Bregma according to the atlas of the mouse brain (25). Riboprobes specific for *WNT-4, GSK-3β, Axin-2, Cyclin-D1* and

DKK-3 mRNA were prepared as described elsewhere (5). Riboprobes specific for *SFRP-2* mRNA were prepared from a 157-bp DNA template, which was generated from *P. sungorus* hypothalamic cDNA by PCR using forward primer 5'-GCC ACG GCA TCG AGT ACC AGA ACA-3' and reverse primer 5'-ACA GGG GCG AAG AGC GAG CAC A-3'. Primers used for amplification of DNA fragments were designed using the Lasergene Primer Select software (DNASTAR, Inc). Amplified DNA fragments were ligated into pGEM-T Easy Vector (Promega Corp), transformed into DH5-α *Escherichia coli*, and sequenced. *In situ* hybridization and analysis were performed as described previously (24). Briefly, cRNA synthesis was facilitated using SP6-polymerase or T7-polymerase as part of a riboprobe synthesis kit (Promega). Slides were fixed, acetylated and dehydrated, followed by overnight hybridization at 58 °C using [^{35}S]-labelled cRNA probes (1–2 × 10^7 cpm/ml). Subsequently, slides were treated with ribonuclease A, desalted and dehydrated. For autoradiography, dried slides were exposed to Amersham Hyperfilm MP (GE Healthcare) together with Amersham [^{14}C]-microscale standard. Images were quantified by measurement of optical density (OD) in a defined area (ARC) using the Image-Pro Plus software (Media Cybernetics) and data were stated as integrated OD (IOD).

Immunohistochemistry

To investigate whether leptin regulates the hypothalamic WNT pathway in a photoperiod-dependent manner, the phosphorylation of the Fz coreceptor LRP-6, as marker of its activation, was analysed. Therefore, Djungarian hamsters were maintained in LD or transferred to SD immediately after weaning. Hamsters received a single intraperitoneal (ip) injection of either leptin (2 mg/kg body weight) or vehicle (0.9 % saline) 15 minutes prior to transcardial perfusion (n = 6–8 animals per each group). Subsequently, immunohistochemistry was performed. Free-floating brain sections were pretreated with 10 % methanol, 1 % NaOH and 1 % H_2O_2 in H_2O for 20 minutes, 0.3 % glycine for 10 minutes and 0.03 % sodium dodecyl sulphate for 10 minutes. Next, sections were blocked with 1 % normal goat serum and 5 % bovine serum albumin in PB-Triton X-100 (0.5 %) for 1 hour, followed by overnight incubation at 4 °C using a rabbit anti-phospho-LRP-6 (Ser1490) antibody (1:1500 in

blocking solution; catalogue no. 2568, Cell Signaling Technology, Inc). On the next day, sections were rinsed and incubated with biotinylated goat-anti-rabbit secondary antibody (1:1000 in blocking solution) for 1 hour, followed by avidin biotin complex solution (Vector Laboratories, Inc) for 1 hour. Finally, the signal was developed by diaminobenzidine solution (Vector Laboratories), giving a grey precipitate. Pictures were taken and immunoreactive cells were counted by two investigators blinded to the treatments.

Statistical analysis

Rhythmicity of each gene expression was analysed using one-way factorial ANOVA with Tukey's honestly significant difference *post hoc* test. To compare phasing and peak expression times, a polynomial fourth order non-linear regression was fitted with GraphPad Prism software (GraphPad Software). The effect of day length on each gene's expression profile was then examined by pairwise comparison. Where polynomial fourth order non-linear regression indicated rhythmicity of a gene, the difference at each time point was analysed by a Student's *t* test. For nonrhythmically expressed genes, the values at the different time points were averaged and the mean expression over 24 hours was compared by Student's *t* test. Coreceptor modification was analysed by two-way ANOVA followed by Holm-Sidak comparison test, as appropriate, using SigmaStat statistical software (Systat Software; Jandel). Results are presented as mean ± SEM, and differences were considered significant if $P \leq 0.05$.

Results

Localisation of WNT signalling gene expression in the brain of Djungarian hamsters

To explore whether WNT signalling is active in the hypothalamus of adult Djungarian hamsters, we analysed expression patterns of genes encoding components of the WNT pathway. By performing *in situ* hybridization, we found that all investigated genes were expressed in the mediobasal hypothalamus, especially in the ARC. Additionally, expression of all genes occurred in extra-hypothalamic regions such as the cortex, the thalamus, and the dentate gyrus (DG) as well as the CA1, CA2 and CA3 fields of the hippocampus (HI). The riboprobe specific for *WNT-4* hybridized also to the choroid plexus. Expression of *GSK-3β* and *Axin-2* was relatively intense in the DG and HI and also occurred in the medial habenula and the ventromedial hypothalamus. Both *Cyclin-D1* and *DKK-3* mRNAs were mainly expressed in the ARC, HI and DG, *DKK-3* additionally in the medial habenula. *SFRP-2* mRNA was intensely concentrated in the thalamus and showed a weaker signal in the ventromedial hypothalamus (Figure 1). Hybridization signals did not occur using the respective sense riboprobes (Figure 1, *DKK-3* sense riboprobe is shown as an example).

Temporal expression pattern of WNT signalling genes in the hypothalamus under long and short photoperiod

Temporal expression patterns of WNT signalling genes in the hypothalamus were examined in LD or SD photoperiod. Non-linear regression analysis revealed a trend for diurnal rhythmicity of *Axin-2* expression (Figure 2a; $P = 0.098$) as well as different amplitude and peak expression times of *Cyclin-D1* and *DKK-3* (Figure 2, b and c) exposing diurnal rhythmicity of both genes. In contrast, no evidence for rhythmic expression of *WNT-4*, *GSK-3β* and *SFRP-2* (Figure 3) over 24 hours was obtained. *Axin-2* gene expression showed similar expression patterns between the two photoperiods. Differential expression of mRNA levels appeared to increase during the dark phase and seemed to decline during the light phase. For both LD and SD, *Axin-2* showed lowest expression levels around the first half of the subjective night (ZT21 and ZT12, respectively).

Cyclin-D1 gene expression during LD showed a trend ($P = 0.098$) towards and during SD a significant diurnal rhythmicity ($P = 0.021$) with decreasing gene expression during the subjective day of the animal and increased gene expression during the subjective night. Consistently, *Cyclin-D1* reached peak values at ZT0 in LD and ZT18 in SD, while trough values were achieved at ZT18 in LD and ZT9 in SD.

DKK-3 showed pronounced high-amplitude rhythmicity in LD ($P < 0.001$) and elevating gene expression throughout the day with peak expression times shortly after the end of the light phase at ZT18 and a trough after the dark phase. Contrarily, in SD, *DKK-3* levels were highest at ZT0 with declining gene expression during the day and elevating gene expression during the night, displaying a generally lower amplitude ($P = 0.023$).

For *Axin-2, Cyclin-D1* and *DKK-3* we found that gene expression was regulated by photoperiod at individual time points. In LD a generally higher expression of *Axin-2* and *Cyclin-D1* was displayed relative to SD, with a trend towards higher *Axin-2* expression at ZT6 ($P = 0.051$) and significantly elevated *Axin-2* expression at ZT9 and ZT12 (Figure 2a; $P < 0.01$). *Cyclin-D*1 expression in LD was elevated at ZT0, ZT3, ZT6 and ZT9 (Figure 2b; $P < 0.05$) compared with SD. *DKK-3* showed a seasonally regulated gene expression with declined expression at ZT3 (Figure 2c; $P = 0.011$) and, conversely regulated, elevated expression at ZT6 ($P = 0.011$) in LD relative to SD.

No diurnal rhythmicity was detected for *WNT-4, SFRP-2* and *GSK-3β* (Figure 3, a–c). However, averaged expression throughout the diurnal cycle revealed that the nonrhythmic genes *WNT-4* and *SFRP-2* were down-regulated in the ARC of SD hamsters compared with LD hamsters by about 20 % and 35 %, respectively (Figure 3, a and b; $P = 0.012$ and $P = 0.005$, respectively). In contrast, there was no effect of photoperiod on gene expression of the pathway-inactivating enzyme *GSK-3β* (Figure 3c; $P = 0.745$).

Effects of photoperiod and leptin on WNT coreceptor activation

After having established that several key components of the WNT pathway are differentially expressed during long and short photoperiod as well as throughout the day, we next tested whether

activation of the WNT pathway at the level of the coreceptor might be dependent on photoperiod. Therefore, we performed immunohistochemistry to detect phospho-LRP-6 (Ser1490)-immunoreactive cells in the ARC of hamsters in LD and SD that were furthermore challenged by ip leptin injections. Two-way ANOVA revealed a statistically significant effect of photoperiod ($P < 0.001$) as well as leptin treatment ($P = 0.011$) on WNT coreceptor activation, however, the effect of leptin was independent of which photoperiod was present ($P = 0.837$; Figure 4). SD hamsters showed a significant decrease in the number of phospho-LRP-6 (Ser1490)-immunoreactive cells of about 40% compared with their littermates in LD. Interestingly, leptin sensitive SD hamsters as well as LD hamsters, which are known to be leptin resistant (1,26), revealed an increase in phospho-LRP-6 (Ser1490)-immunoreactive cells of about 45% and 30%, respectively, in response to ip leptin compared with hamsters that received vehicle.

Discussion

The WNT pathway has been well characterized in embryogenesis and tumorigenesis. However, recent data suggested that this pathway has a much broader function in the central nervous system than initially thought. Accumulating evidence suggests that it is involved in adult neurogenesis (7), the remodelling of the adult hypothalamus (8) and the neuroendocrine control of metabolism (5,9). In the present study, we investigated the primary effects of photoperiod on WNT signalling in the seasonal mammal *Phodopus sungorus* in order to unravel its role in long-term seasonal changes in energy metabolism. Furthermore, we characterized whether mRNA expression is regulated in a diurnal manner.

We demonstrated that all investigated genes involved in the WNT pathway are expressed in the hypothalamic ARC, a key region in neuronal control of body weight and food intake. However, it is important to note that expression of WNT genes also occurred in extra-hypothalamic brain regions such as the cortex, thalamus and hippocampus. The change in the mRNA expression of several WNT components (*WNT-4, Axin-2, Cyclin-D1, DKK-3* and *SFRP-2*) during the differential photoperiods indicates that central WNT signalling may play an important role in the seasonal regulation of body weight and food intake in adult Djungarian hamsters. We have previously demonstrated that WNT signalling is active in the adult murine brain (5). The expression of key members of the WNT pathway in several brain regions of two rodent species, together with the finding that WNT genes are differentially expressed in response to changing photoperiod in *Phodopus sungorus* corroborates the importance of this pathway in the neuronal control of metabolism and its prominence in the adult brain.

We focussed on WNT-4 and WNT-7a (27) as ligands for the WNT pathway, since both have been shown to be implicated in neural development, such as anterior-posterior guidance of commissural axon growth (28), maturation of synapses (29), neuronal differentiation (30) and central nervous system vascularisation (31). Unfortunately, we could only analyse gene expression data for *WNT-4* because *WNT-7a* expression was below the detection limit of the very sensitive *in situ* hybridization using radiolabelled probes. Gene expression of the ligand *WNT*-4, which activates the WNT pathway

(32), was up-regulated in the ARC of LD relative to SD hamsters. This suggests an improved receptor activation of the WNT pathway in the ARC of LD-acclimated hamsters. Together with our finding that the WNT target genes *Axin-2* (33) and *Cyclin-D1* (34) were increased at certain time points throughout the diurnal rhythm in LD relative to SD, these data imply enhanced activation of the WNT signal transduction pathway in this photoperiod. *Cyclin-D1* is a prominent mediator of mammalian cell growth (35,36) and furthermore, WNT/β-catenin-mediated Cyclin-D1 expression directly leads to enhanced cell proliferation (34). Thus, our data indicate increased neural cell proliferation in LD.

A crucial regulatory enzyme of WNT signalling is GSK-3β because ligands binding to WNT receptors lead to inactivation of GSK-3β and thereby activation of the pathway. We did not find regulation of GSK-3β by photoperiod on the level of gene expression. However, GSK-3β activity is regulated by post-translational modification and the formation of an Axin-including degradation complex (37). Therefore, gene expression data of this particular member of the WNT pathway are not very informative and we attempted to detect phosphorylated GSK-3β, which would allow us to determine the activity of this enzyme. Unfortunately, the readily available commercial antibodies to detect phospho-GSK-3β did not cross-react with hamster brain tissue.

SFRP-2 mRNA was significantly up-regulated in the ARC of LD-acclimated hamsters compared with SD-acclimated hamsters. This is consistent with our previous study that showed that *SFRP-2* gene expression is increased in the hypothalamus of photoperiod-responsive F344 rats under LD relative to SD conditions (8). Some studies have shown that SFRP-2 acts as an antagonist of the WNT pathway via interaction with WNT ligands to prevent them from binding to Fz receptors (38-40) and this appears contradictory to activated WNT signalling in LD. However, the WNT-antagonizing effect of SFRP-2 has not been clearly established because, in contrast, Yoshino *et al*. demonstrated that SFRP-2 inhibits other SFRPs to promote WNT activity (41). Interestingly, there is evidence that WNT-4 does not only induce SFRP-2 expression in particular tissues, but SFRP-2 also binds WNT-4 and enhances its signal transduction (39,41). The mutual up-regulation of both genes in the ARC of LD hamsters might thereby have a synergistic effect, which might cause the up-regulation of the targets *Axin-2* and *Cyclin-D1* observed in this study.

In a previous study, we observed a marked photoperiodic response of *DKK-3* with elevated gene expression during LD in F344 rats (8,10). In the present study, *DKK-3* gene expression was profoundly coupled to the diurnal rhythm and regulated by photoperiod. Whether *DKK-3* was elevated or reduced in LD relative to SD was dependent on the time of day, suggesting that the diurnal control of this WNT-related gene overrides any photoperiodic regulation. In LD hamsters *DKK-3* gene expression was tightly coupled to the light/dark phase. It continuously increased throughout the light phase followed by a decrease during the dark phase. DKK-3 is a member of the DKK family that inhibits WNT signalling by binding to the LRP-5/6 coreceptors (42). In line with the WNT inhibitory function of DKK-3, expression of the WNT target genes *Axin-2* and *Cyclin-D1* declined during the animals' subjective day and elevated during their subjective night in LD. The diurnal regulation of *DKK-3* on the one hand and reciprocal *Axin-2* and *Cyclin-D1* gene expression on the other hand implies that diurnal regulation of *DKK-3* in LD hamsters might have a yet unknown physiological function to regulate WNT target genes in a circadian manner that is restricted to this photoperiod. Diurnal *DKK-3* gene expression in SD did not affect *Axin-2* and *Cyclin-D1* mRNA in the same manner as in LD. Surprisingly, the precise function of DKK-3 in WNT signal transduction has not yet been established. Although some studies have shown that DKK-3 has no influence on LRP-5/6 activation or nuclear β-catenin accumulation (43,44), others revealed effects on cytoplasmic β-catenin levels (45,46). Moreover, an implication of DKK-3 in non-canonical WNT/c-Jun N-terminal kinase (JNK) signal transduction is being discussed (47,48).

The diurnal rhythmicity of several WNT genes in the ARC might be suggestive for an interaction of the WNT pathway and the circadian clock. Although the master circadian clock resides in the SCN, a food-entrainable oscillator has been proposed to be located in the ARC (49). In fact, WNT signal transduction was shown to be modulated by the clock gene brain and muscle Arnt-like 1 (Bmal1) with declined WNT signalling after attenuation of Bmal1 function (50). Because Bmal1 is known to be a prominent regulator of genes that control metabolism (51), these data support the potential role of WNT signalling in the control of metabolic processes in the Djungarian hamster.

Initiation of the canonical WNT pathway requires activation of both the Fz receptor and the LRP-5/6 coreceptor. LRP-6 is activated by phosphorylation at Ser1490 (12), leading to recruitment of Axin to the intracellular domain of the coreceptor (52-54) and subsequent inhibition of GSK-3β. The number of phospho-LRP-6 (Ser1490)-immunoreactive cells was enhanced in the ARC of LD hamsters compared with SD hamsters. Peripheral administration of the adipokine leptin induced an increase of phospho-LRP-6 (Ser1490)-immunoreactivity in LD as well as in SD hamsters. This is consistent with our previous finding in obese leptin deficient $Lep^{ob/ob}$ mice, which exhibited increased phospho-LRP-6 (Ser1490) immunoreactivity and *Axin*-2 and *Cyclin-D1* gene expression after leptin injection (5). Notably, in the brain regions in which we detected WNT gene expression, the long form of the leptin receptor (Lep^{Rb}) is also widely expressed in the Djungarian hamster and other rodent models (55-58). The coexistence of both leptin and WNT signalling in these brain regions indicates that the two pathways interact. By corroborating our findings from mice (5) in hamsters, we provide the first evidence of a novel, previously unknown central effect of leptin, ie, leptin is capable of activating the WNT coreceptor LRP-6 across different species, revealing a potentially very important physiological regulation of the WNT pathway by leptin. This activation occurred at the level of the coreceptor LRP-6, which suggests that leptin activates the canonical WNT pathway. Future studies, however, are required to determine whether this hormone also affects non-canonical WNT signalling such as the planar cell polarity and Ca^{2+} pathways (59,60). Intriguingly, leptin enhanced LRP-6 activation in hamsters from both photoperiods. During LD photoperiod hamsters are resistant to exogenous leptin in terms of its catabolic effect (1), and also in terms of its ability to activate the signal transducer and activator of transcription 3 (STAT3) (61). The transcription factor STAT3 is part of the best characterised Janus kinase 2-STAT3 leptin signalling pathway. Also, gene expression of the suppressor of cytokine signalling 3, which inhibits leptin signalling, was increased in LD compared with SD conditions (26,62,63). It is plausible that leptin activates the WNT pathway at the level of LRP-6 independent of Janus kinase 2-STAT3 signalling. Although leptin levels are elevated in LD compared with SD, in neither photoperiod circadian regulation of mean serum leptin levels was detected over a 24 hour profile in Djungarian hamsters (64,65). This suggests that the diurnal

regulation of WNT target genes and *DKK-3* might be independent of circulating leptin. It has been reported, however, that gene expression of Lep^{Rb} is regulated in a circadian manner in the ARC of hamsters exposed to LD (64). This indicates that Lep^{Rb} might be involved in circadian regulation of WNT signalling components. Circadian changes in Lep^{Rb} expression were confined to LD hamsters (64), suggesting that leptin sensitivity is under the control of the circadian rhythm in LD which counteracts to the perception that LD hamsters are generally leptin resistant relative to SD hamsters. The necessity to further assess the role of leptin sensitivity in seasonal body weight regulation is further supported by our finding that leptin activated LRP-6 in both photoperiods. Regulation of leptin sensitivity might be fine-tuned at different levels in the leptin receptor signalling cascade.

In LD hamsters, high circulating levels of leptin in combination with the synergistic effect of WNT-4 and SFRP-2 might potentiate induction of phosphorylation of LRP-6, probably preventing the Axin-GSK-3β complexation and inhibiting GSK-3β. This would facilitate the expression of WNT target genes such as *Axin-2* and *Cyclin-D1*. During SD, lower leptin levels in combination with reduced WNT-4 and SFRP-2 would explain the lower basal activation of LRP-6 relative to LD. Less activation of WNT signalling would attenuate the inhibition of GSK-3β which as a negative WNT pathway regulator would lead to further reduction of WNT signalling and reduced target gene expression (Figure 5).

In conclusion, we provide strong evidence that WNT signalling is involved in the seasonal as well as the diurnal regulation of metabolism in the hypothalamus of the Djungarian hamster. The expression pattern of the investigated genes involved in WNT signalling and the post-translational modification of the WNT coreceptor strongly indicate that this pathway is functionally impaired in the hypothalamus of Djungarian hamsters during SD and reinstated during LD photoperiod. Furthermore, these data corroborate our previous observation of leptin's ability to activate the WNT signal transduction in mice and imply a fundamental importance of WNT signalling in metabolic control (5).

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Figure 1. Genes encoding members of the WNT pathway are expressed in the brain of Djungarian hamsters. Genes encoding for the WNT ligand WNT-4, the key enzyme GSK-3β, the WNT target genes *Axin-2* and *Cyclin-D1* and the antagonists SFRP-2 and DKK-3 were detected by *in situ* hybridization with antisense [^{35}S]-labelled riboprobes. All investigated genes were expressed in the ARC of the hypothalamus. Additionally, some expression occurred in the cortex, hippocampus and thalamus. Representative for all respective sense riboprobes an image for *DKK-3* is shown. Inserts depict binding of the riboprobes to the ARC.

568 **Figure 2.** Temporal expression profiles of WNT signalling genes in the ARC of Djungarian hamsters
569 acclimated to either long day (LD, □) or short day (SD, ▲) photoperiod. *Axin-2* (a), *Cyclin-D1* (b)
570 and *DKK-3* (c) were rhythmically expressed in both photoperiods. The left panels depict
571 autoradiographs of representative coronal brain sections from time points when temporal gene
572 expression was different between LD and SD; inserts in the left panels show binding of riboprobes to
573 the ARC. The right panels show line charts of quantified signals in the ARC from three to four
574 sections per animal. Open and solid bars at the top of each graph represent light and dark periods,
575 respectively. Data are presented as means ± SEM (n = 2–6 at each time point). *$P \leq 0.05$, **$P \leq 0.01$
576 reveal significantly different time points of gene expression between LD and SD. IOD, integrated OD.
577

578 **Figure 3.** Temporal expression profiles of WNT genes of which no rhythmicity was detected over
579 24 hours. Averaged expression over 24 hours of *WNT-4* (a) and *SFRP-2* (b) was elevated in LD
580 compared with SD, whereas *GSK-3β* gene expression (c) was not affected by photoperiod. The left
581 panels show line charts of quantified signals in the ARC from three to four sections per animal. The
582 right panels show bar charts of averaged expression throughout the diurnal cycle with autoradiographs
583 of representative coronal brain sections from LD and SD; inserts depict binding of riboprobes to the
584 ARC. Data are presented as means ± SEM; $*P \leq 0.05$, $**P \leq 0.01$. IOD, integrated OD.
585

Figure 4. Photoperiod and leptin administration had a significant effect on WNT coreceptor activation. Immunoreactivity of phospho-LRP-6 (Ser1490) was increased in the ARC of LD compared with SD hamsters. Independent of photoperiod, leptin (2 mg/kg) administered intraperitoneally led to a significant increase of phospho-LRP-6 (Ser1490)-immunoreactive cells in the ARC compared with vehicle. Upper panels depict representative coronal brain sections of each group, lower panels show bar charts of quantified phospho-LRP-6-immunoreactive cells in the ARC from three sections per animal. Data are presented as mean percentage values of LD control ± SEM; $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

Figure 5. Model proposing the seasonal regulation of the WNT pathway in the hypothalamus of the Djungarian hamster. (a) During LD, when leptin levels are elevated, phosphorylation of LRP-6 is increased, which might be mediated via synergistic action of up-regulated WNT-4 and SFRP-2 on the one hand and leptin on the other hand. LRP-6 is known to phosphorylate and thereby inactivate GSK-3β. This overrides the inhibitory effect of GSK-3β on WNT target gene expression and therefore increases *Axin-2* and *Cyclin-D1* mRNA. (b) During SD, when leptin levels are low, reduced levels of WNT-4, SFRP-2 and leptin fail to activate LRP-6, leading to enhanced activity of GSK-3β, which in turn would lead to reduced WNT target gene expression. The putative LRP-5/6 antagonist DKK-3 is differently regulated at individual time points in LD and SD, yet its role in WNT signalling is still unclear. Furthermore, the gene expression of *DKK-3* as well as the WNT target genes *Axin-2* and *Cyclin-D1* was under diurnal rhythmicity, indicated by Θ.